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Mass Spectrometric Analysis of Tobacco-Specific Hemoglobin Adducts

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Hemoglobin adducts of the common metabolite of the tobacco-specific nitrosamine (TSNA) 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB) and of 4-aminobiphenyl (4-ABP) were quantified in blood samples from smokers and nonsmokers to test their suitability for biomonitoring tobacco smoke exposure. Additionally, TSNA adducts were measured in nasal snuff users. Mild alkaline treatment of hemoglobin releases 4-ABP and HPB, which were analyzed in parallel by capillary gas chromatography with electronic impact or negative ion chemical-ionization mass spectrometry (EI- or NICI-GC-MS). Samples of snuff users showed high levels of HPB adducts not correlated with the amount or type of snuff used. HPB concentrations in smokers and nonsmokers, however, were much lower, with no group-specific differences detectable. In contrast, 4-ABP adduct levels were much higher in smokers than in nonsmokers, confirming the significant difference between these two groups reported by others.

Introduction

Upon metabolic activation, constituents of tobacco and tobacco smoke such as the aromatic amine 4-aminobiphenyl (4-ABP) and the tobacco-specific nitrosamines (TSNA) *N'*-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) yield hemoglobin adducts that have been suggested as powerful biomarkers for exposure. Both compound classes are strong animal carcinogens and possibly involved in urinary bladder and lung tumorigenesis of smokers, respectively (*1,2*). Additionally, TSNA are considered to be a causative factor for tumors in the oral cavity of snuff dippers (*1*). High adduct levels of 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB) have been detected in hemoglobin samples from snuff dippers. However, the differences between smokers and nonsmokers were not as marked as expected (*3*). 4-ABP has been considered to be less specific for exposure to tobacco and/or tobacco smoke because of other possible environmental sources. Nonetheless, a highly significant difference was observed in 4-ABP adduct concentrations between smokers and nonsmokers (*2*). Therefore, it was decided to determine hemoglobin adducts of both classes of chemicals to better establish their suitability for biomonitoring tobacco smoke exposure.

Analysis of Human Blood for Hemoglobin Adducts

The protocol as described by Carmella et al. (*3*) was modified according to the method described by Stillwell et al. (*4*) for analysis of adducts formed by aromatic amines, to enable parallel detection of adducts formed both by HPB and 4-ABP. The principle of the procedure used in our laboratory is outlined in Figure 1.

To prevent contamination and minimize background values, disposable polypropylene tubes are used for the first steps. After eluting the concentrated solutions from C-18-Bond Elut extraction tubes, only glassware could be used.

Red blood cells were isolated from whole blood by centrifugation and repeated washing with 0.9% NaCl solution. Erythrocytes were hemolyzed by adding three volumes of ice-cold water. After cooling to 0°C for 15 min, 1/3 volume of phosphate buffer (0.67 m, pH 6.6) was added to adjust ionic strength. Cell membranes were spun down at 18000 rpm (30 min), and the supernatant was dialyzed against water for 48 hr. The resulting hemoglobin solution was frozen at -20°C until analysis.

Before alkaline hydrolysis, the internal standards 4-hydroxy-1-(3-pyridyl)-[2,3,3,4,4-d_5]-1-butanone (d_5-HPB) and 4-amino-4'-fluorobiphenyl (F-4-ABP) were added and the adducts liberated from hemoglobin by hydrolysis with 1/10 volume of 1 N NaOH. The solution was sonicated for 1 hr at room temperature, protein precipitated by addition of 20% (w/v) $(NH_4)_2SO_4$ and collected by centrifugation. To avoid the multiple extraction procedure employed by Carmella et al. (*3*), the supernatant was applied to C-18-Bond Elut extraction tubes. The sample was pulled through with a vacuum. Columns were washed twice with water and retained HPB and 4-ABP as well as the internal standards are eluted in a small volume ($\sim$1 mL) acetonitrile.

Test runs with blank samples have shown that beginning with this step, polypropylene tubes will give strong contamination in GC-MS analysis, likely stemming from compounds extracted from the plastic by organic solvents.

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Collection of erythrocytes from blood samples (≈20 mL)
hemolysis by addition of ice-cold distilled water
(store at -20°C if desired)

↓

Hb quantification

↓

addition of internal standards (D_5-HPB and F-4-ABP)
protein cleavage by sonication in 0.1 N NaOH for 1 hour

↓

protein precipitation with 20% (w/v) ammonium sulfate
centrifugation

↓

clear supernatant over C18-Bond Elut (1 mL bed volume),
wash with 2 volumes distilled water

↓

elution of HPB with <1 ml acetonitrile,
evaporate acetonitrile, resuspend sample in
150 µL hexane, 400 µL CH_2Cl_2, 400 µL triethylamine
(1 M in hexane)

↓

add 2 µL PFBC and 8 µL PFPA for derivatization,
heat at 60°C for 2 hours,
evaporate solvent

↓

resuspend sample in 500 µL ethanol with pentano- and hexanophenone as retention time markers for purification on HPLC
(12.5 cm, C18; eluent 55% acetonitrile/water, 0.7 mL/min)
retention time of HPB ≈14 min, of 4-ABP ≈19 min

↓

extract sample three times with 0.5 mL hexane
evaporate solvent completely
resuspend in 20 µl hexane, analyze 2 µL by NICI-GC-MS

FIGURE 1. Analytical procedure for the analysis of hemoglobin adducts.

Acetonitrile was evaporated to dryness under a stream of N_2 and the residue derivatized with a mixture of pentafluorobenzoyl chloride (PFBC) and pentafluoropropionic anhydride (PFPA). These reagents are especially suitable for highly selective and sensitive analysis by negative ion chemical ionization (NICI)–GC–MS of HPB and 4-ABP, respectively. After incubation at 60°C for 2 hr the sample was evaporated completely, resuspended in 500 µL ethanol containing pentano- and hexanophenone as retention time markers, and purified by isocratic HPLC (C-18 column, 50% acetonitrile/water, flow rate 0.7 mL/min). The substances eluting between the markers at 12 and 20 min were collected, extracted three times with hexane, and the organic phase concentrated to 20 µL in small glass vials.

Aliquots of 2 µL were injected onto a capillary column (UP2, 30 m) for NICI-GC-MS analysis in single ion mode (SIM) using m/e 359 and 363 as characteristic ions for HPB and d_5-HPB and 295 and 313 as characteristic ions for 4-ABP and F-4-ABP. Samples from snuff dippers were analyzed by electron impact (EI)-GC-MS using m/e 121 and 122 for HPB and d_5-HPB.

Results and Discussion

The major result of this study is that hemoglobin adducts from two different chemical classes, aromatic amines and nitrosamines, can be determined in a combined analytical procedure. This allows the determination of two independent markers for exposure to tobacco smoke from the same sample. Tests have shown that cross-reactivity of HPB with PFPA and 4-ABP with PFBC is low and does not disturb the analysis of either compound.

Table 1. HPB and 4-ABP levels in nonsmokers, smokers, and nasal snuff users.[a]

Status	Subject	Sex	HPB, fmole/g Hb	4-ABP, fmole/g Hb
Nonsmoker	1	F	130	ND
	2	M	247	23
	3	M	ND	NA
	4	F	ND	NA
Smoker (cigarettes/day)				
5	1	M	168	749
15	2	M	ND	403
5	3	M	182	3468
20	4	M	ND	NA
15	5	M	124	NA
Nasal snuff user (g/week)				
60	2	M	1010	NA
35	3	M	2200	NA
28	4	M	690	NA
10	5	M	830	NA
20	6	M	630	NA
50	7	M	ND	NA
20	8	M	1360	NA
55	10	M	800	NA
10	11	M	1770	NA
125	12	M	1020	NA
50	13	M	1040	NA
35	14	M	1010	NA
10	15	M	720	NA
10	16	M	1650	NA

Abbreviations: HPB, 4-hydroxy-1-(3-pyridyl)-1-butanone; 4-ABP, 4-amino biphenyl; ND, not detected; NA, not analyzed.

[a]Nonsmoking status was given only by individual's declaration. Nasal snuff users use different kinds of nasal snuff.

The number of analyses done so far is still too small to draw definitive conclusions. As shown in Table 1, high levels of HPB were detected in the blood from users of nasal snuff. This is in agreement with the results of Carmella et al. (*3*), who found high adduct levels in snuff dippers. The much higher concentrations in snuff dippers as compared to smokers cannot be explained easily by higher uptake of preformed NNN and NNK. Because of the rather low TSNA concentrations in nasal snuff from Bavarian manufacturers as compared to different brands of U.S. snuff (*5*) and the use of rather low amounts of tobacco this difference is even more remarkable in the case of our snuff users.

The lack of significant differences in HPB levels between smokers and nonsmokers confirm the results of Carmella et al. (*3*). The reason for the overlapping of the adduct values in these groups is not known. Interindividual differences in biotransformation of TSNA or in endogenous TSNA formation from precursor alkaloids are plausible explanations at the moment.

Adducts of 4-ABP have been successfully used for biomonitoring the exposure to tobacco smoke (*2*). The data in the present study confirm the suitability of this adduct. Future experiments are designed to separate HPB and 4-ABP by HPLC before derivatization, using automatized on-line precolumn enrichment (*6*), thus excluding any difficulties that could arise from cross-reactivity.

In conclusion, we have shown that the simultaneous determination of human hemoglobin adducts arising from aromatic amines as well as from tobacco-specific nitrosamines can be easily accomplished.

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